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Occluded and Nonoccluded Nuclear Polyhedrosis Virus Grown in *Trichoplusia ni*: Comparative Neutralization, Comparative Infectivity, and In Vitro Growth Studies

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Nuclear polyhedrosis virus infections of lepidopteran cells often result in the production of both occluded and nonoccluded virus. The characterization of these two different forms has been the subject of several papers. We have divided the nonoccluded virus (NOV) category further into plasma membrane-budded nonoccluded virus (PMB-NOV), intracellular NOV, and hemolymph-derived NOV, and have done additional studies investigating the differences between these nonoccluded forms and the alkali-liberated forms from occlusions of the nuclear polyhedrosis viruses of *Autographa californica* and *Rachiplusia ou*. The methods used to discern differences and similarities among the forms were serological, biochemical, and visual, all related to their biological activity. Neutralization studies revealed that alkali-liberated virus and PMB-NOV had both similar and different antigens. Antisera raised against alkali-liberated virus from occlusions neutralized the alkali-liberated form of the virus, but did not neutralize the intracellular or extracellular nonoccluded forms. Antisera raised against the TN-368-13 PMB-NOV, however, neutralized the alkali-liberated forms as well as all forms of the NOV. Adsorption of this antisera with alkali-liberated virus did not diminish the neutralization titer against the nonoccluded forms, thus confirming the antigenic differences between the alkali-liberated and nonoccluded forms of the virus. Physical-infectious particle ratio calculations indicated that the PMB-NOV of *Autographa californica* are about 1,900-fold more infectious than the single-nucleocapsid-per-envelope alkali-liberated particles and about 1,700-fold more infectious than the multiple-nucleocapsid-per-envelope particles, as assayed in vitro. In addition, a study of viral growth kinetics monitored concurrently with the appearance of polyhedra showed that PMB-NOV production is shut down with the onset of polyhedron formation.

It is likely that nuclear polyhedrosis viruses (NPVs), with genomes of approximately 1×10^8 molecular weight (26), will eventually be considered to be among the most complex of animal viruses. Their complexity is just beginning to unfold as increasing numbers of investigators turn to the study of this very interesting and potentially economically useful group of biological agents.

Among the observations that thus far attest to the nonsimplistic nature of the NPVs is that during replication at least three and possibly four modified infectious forms can be produced. One of these is the occluded form, which gains its envelope by probable de novo synthesis in the nucleus and then becomes incorporated into an inclusion body there (13, 16, 24). A second modified form occurs when particles bud from the inner nuclear membrane (13, 16, 19), and a third occurs when unenveloped particles bud through the plasma membrane (13, 16, 19, 29). A fourth modification is the absence of membrane, i.e., the unenveloped particles (nucleocapsids) as they occur both intranuclearly and intracytoplasmically (13). It is reported that the occluded, the budded, and the intracellular viruses are all infectious, though it is not known whether it is the enveloped or unenveloped particle, or both, that is the intracellular infectious component. Indeed, there have been a number of studies that focused on characterizing the nonoccluded infectious agent from infectious hemolymph and/or cell culture medium (5, 10, 15, 17, 23, 29, 32). In most of the in vitro studies, however, the nonoccluded virus (NOV) was collected from the cell culture medium after extensive cytolysis had occurred, and thus it is probable that the intracellular enveloped, unenveloped, and budded forms were all included under the somewhat broad classification of nonoc-

cluded virus (10, 17). Understandably, considerable confusion resulted as to the nature of the nonoccluded particles (29). A similar situation in vivo could account for some of the contradictory results obtained in studies of the hemolymph-derived particles; i.e., the nonoccluded particles in the hemolymph could include both budded and intracellular particles released by cytolysis (15, 23, 32).

It is reasonable to expect that some of these different infectious forms of NPV could have different roles in the continuance of the complicated infection cycle of the virus in nature. Perhaps the first step in examining this notion is to document actual differences that exist among these forms. In doing this, however, careful attention should be paid to the relative purity of the form(s) being studied. It is anticipated that a comprehensive study of these virus- and/or host-induced modifications of virus structure could reveal information relative to host cell specificity and/or tissue tropisms.

In an earlier study, Summers and Volkman (29) compared a number of different properties of culture-derived, plasma membrane-budded (PMB) *Rachiplusia ou* and *Autographa californica* NPV (Ro*M*NPV and Ac*M*NPV; *M* is used to designate many nucleocapsids common to a single envelope), hemolymph-derived Ac*M*NPV and Ro*M*NPV, and larvae-derived, alkali-liberated Ac*M*NPV and Ro*M*NPV. Their study showed that PMB-NOV were loosely enveloped single nucleocapsids with peplomers on their surfaces. Similarly, the infectious hemolymph-derived virions were enveloped and had surface projections. Both the PMB-NOV and the hemolymph-derived virions banded on a sucrose density equilibrium gradient as a single major band at 1.17 to 1.18 g/ml, with a minor band (attributed to nucleocapsids) at a density greater than 1.3 g/ml. In contrast, the virions released from inclusions were for the most part packaged in multiple-nucleocapsid-per-envelope units, which appeared as multiple bands on sucrose density gradients. The buoyant density, which varied with the number of nucleocapsids per envelope, ranged from 1.20 g/ml for the single nucleocapsids per envelope to 1.25 g/ml for more than nine nucleocapsids per envelope. The envelopes, too, were relatively tight fitting and were devoid of surface projections.

In this paper we continue the investigation of the differences between alkali-liberated PMB-NOV and other nonoccluded forms of Ro*M*NPV and Ac*M*NPV. Neutralization studies reveal that alkali-liberated and nonoccluded forms are antigenically different. Antisera raised against gradient-purified Ac*M*NPV *l*arvae-derived *o*ccluded *v*irions that have been *a*lkali *l*iberated (LOVAL) will not neutralize nonoccluded homologous virus from infectious hemolymph, alkali- and untreated PMB-NOV from cell culture medium, or nonoccluded intracellular virus from cultured cells or infected fat bodies. It will neutralize both Ac-*M*NPV and Ro*M*NPV LOVAL, however. Conversely, antisera to Ro*M*NPV LOVAL will not neutralize nonoccluded Ro*M*NPV but will neutralize both Ro*M*NPV and Ac*M*NPV LOVAL. Antisera raised against Ac*M*NPV PMB-NOV neutralize not only all nonoccluded forms of both Ac*M*NPV and Ro*M*NPV tested, but the LOVAL forms as well. Adsorption of these antisera with Ac*M*NPV LOVAL removes all of the anti-LOVAL neutralization activity, but does not diminish the anti-NOV neutralization titer, indicating that whereas the alkali-liberated and nonoccluded forms of the virus share some antigenic determinants, those involved in the neutralization of the two different forms are different.

Physical-infectious particle ratio calculations indicate that PMB-NOV Ac*M*NPV from the culture medium of TN-368-13 cells is 1,700-fold more infectious than multiple-nucleocapsid-per-envelope LOVAL and 1,900-fold more infectious than single-nucleocapsid-per-envelope LOVAL, as assayed in vitro.

Further, studies of virus growth kinetics in which the appearance of different stages of cytopathic effect (CPE) were monitored concurrently with the appearance of extracellular virus suggest that the formation of polyhedra and the shutdown of virus budding from the plasma membrane are related events.

MATERIALS AND METHODS

Cell culture. The continuous cell line of *Trichoplusia ni*, TN-368, was obtained in the 57th passage from W. F. Hink of Ohio State University in June 1974. Since then the cells have been passed routinely three times per week in disposable plastic tissue culture flasks, with the initial concentration being 1×10^5 to 2×10^5 cells per ml. Daughter cell lines were developed from isolated single cells of TN-368 (Volkman and Summers, *in* Proc. IV Int. Conf. Invert. Tissue Culture, in press), and these cloned lines were handled in the same manner as TN-368. The cultures were grown at 28°C, and the medium used was TNM-FH (11). Antibiotics were not used for routine maintenance of the cells.

For one experiment, virus was grown in a continuous line of *Spodoptera frugiperda* pupal ovarian cells (J. L. Vaughn, unpublished data). This cell line was obtained for use in our laboratory from H. Stockdale. The cells are routinely passed three times per week and are maintained in BML-TC/10 medium (G. R. Gardiner and H. Stockdale, Abstr. Proc. VI Annu. Meet. Soc. Invertebr. Pathol., p. 20, 1973).

Growth kinetics of infected and uninfected cells. Log-phase TN-368 clone 10 (TN-368-10) and TN-368 clone 13 (TN-368-13) cells in new 25-ml plastic cell culture flasks were infected in a total volume of 1 ml with Ac*M*NPV NOV at multiplicity of infection of 10 to 20. After 1-h adsorption, 4 ml of TNM-FH plus antibiotics were added. Periodically, viable cell counts were made by using trypan blue and a hemocytometer.

Virus. Initially, Ac*M*NPV was supplied by P. V. Vail and W. F. Hink in the form of infectious tissue culture medium. TN-368 cells infected with this medium yielded polyhedra that were in turn used to infect *T. ni* larvae for the production of infectious hemolymph. Ro*M*NPV was obtained in the occluded form from C. Y. Kawanishi. *T. ni* larvae infected with the polyhedra yielded infectious hemolymph, which was used subsequently to infect TN-368 cells for the production of NOV.

Hemolymph used in the neutralization experiments was collected from infected *T. ni* larvae by removing one or two prolegs and catching the resultant drops of infectious fluid in a test tube containing TNM-FH plus antibiotics (200 μg of penicillin and streptomycin per ml and 5 μg of amphotericin B [Fungizone] per ml).

Preparations of LOVAL used as antigens for generating antisera as well as in neutralization experiments were made in the following way. The virions were liberated from gradient-purified polyhedra by incubating the polyhedra for 10 min at 28°C in 0.1 M Na_2CO_3 + 0.05 M NaCl, pH 10.9 (28). The preparations were then immediately layered on sucrose gradients made in 0.01 M Tris buffer (pH 7.8)-0.01 M EDTA (ranging in density from 1.18 to 1.25 g/ml) and centrifuged for 2 h at 24,000 rpm (SW27 rotor). The distinct bands of one through many nucleocapsids per envelope were collected and pooled (29). For some experiments, the band containing one nucleocapsid per envelope was kept separate from the rest.

Virus from the fat body of Ac*M*NPV-infected larvae was collected in the following way. The infected fat bodies were removed from five larvae and homogenized in TNM-FH plus antibiotics with a glass homogenizer. The mixture was centrifuged at 2,100 $\times$ *g* for 15 min, and the supernatant fluid was decanted and filtered through a 0.18- to 14-μm microfiberglass filter. The resultant infectious preparation was used in neutralization studies.

Nonoccluded intracellular virus from TN-368-13 was obtained simply by homogenizing a suspension of Ac*M*NPV-infected cells, which had been previously pelleted and rinsed twice, in TNM-FH. The disrupted cell suspension was cleared of heavy and large debris by centrifugation at 4,000 $\times$ *g* for 15 min. The supernatant fluid was used in the neutralization experiment.

PMB-NOV was obtained from the culture medium of synchronously infected TN-368-13 (or *S. frugiperda*) cells in advance of cytolysis 36 to 48 h after infection (PMB-NOV will refer to TN-368-13-derived virus unless specifically referred to as otherwise). Cells were pelleted by centrifugation at 300 $\times$ *g* for 15 min (to minimize cell disruption), and the supernatant fluid was further clarified by centrifugation at 4,000 $\times$ *g* for 15 min. This 4,000 $\times$ *g* supernatant fluid constituted the crude virus preparation, which was used in the neutralization experiments. For some studies (e.g., quantification of physical particles and the generation of antisera), the preparation was further purified as follows. The 4,000 $\times$ *g* supernatant fluid was centrifuged at 12,000 $\times$ *g* for 45 min to pellet the virus. The pellets were resuspended in 0.01 M Tris (pH 7.8)-0.01 M EDTA and layered on sucrose gradients in that same buffer, ranging in density from 1.1 to 1.3 g/ml. The gradients were centrifuged at 100,000 $\times$ *g* for 4 h or 358,000 $\times$ *g* for 1 h at 4°C. The 1.17- to 1.18-g/ml region of the gradients containing the virus was collected and held for experimentation (29).

Physical-infectious particle ratio determinations. The number of physical particles present in a preparation was calculated by using the following values. The molecular weight of the genome of Ro*M*NPV and Ac*M*NPV was estimated to be 0.9×10^8 to 1×10^8, based upon sedimentation relative to T4 DNA (26). The DNA-protein ratio of a preparation of purified Ac*M*NPV single-nucleocapsid-per-envelope LOVAL was determined to be 0.20, which is in close agreement with the value reported for *T. ni* granulosis virus by Summers and Paschke (27). The DNA-protein ratio of Ac*M*NPV multiple-nucleocapsids-per-envelope LOVAL was determined to be 0.30; that of Ac*M*NPV PMB-NOV was determined to be 0.19. The DNA values were determined by the diphenylamine method (3), and the protein values were determined by the method of Lowry et al. (18).

The number of physical particles in a given preparation of highly purified Ac*M*NPV single-nucleocapsid-per-envelope LOVAL, then, was calculated as follows. For every microgram of protein there is 2×10^{-7} g of DNA. This amount (2×10^{-7} g) of DNA divided by 1×10^8 g per mol (using 10^8 as the molecular weight) gives 2×10^{-15} mol of DNA per μg of protein, and this times the Avogadro constant, 6.02×10^{23} particles per mol, gives 1.20×10^9 particles per μg of viral protein. Using the same method of calculation and by substituting in the appropriate numbers, it can be determined that there are approximately 1.8×10^9 particles per μg of protein with LOVAL bundles and 1.1×10^9 particles per μg of protein of PMB-NOV.

The infectivity of the freshly purified unfrozen preparations was determined by the plaque assay, as described below.

Plaque assay of infectious virus. For the plaque assay (12, 31), long-phase TN-368 cells were seeded into 30-mm Corning plates at 3.5×10^5 cells per plate in 2 ml of medium. Cells were allowed to attach for 1 to 2 h. The medium was carefully removed, and the cells were inoculated with a 0.1-ml sample (per plate) containing between 50 and 250 PFU. Plates were rocked every 10 min for 1 h and then overlaid with 0.9% methylcellulose in TNM-FH containing 200 μg each of penicillin and streptomycin per ml and 5 μg of Fungizone per ml. All assays were done in duplicate.

Viral growth kinetics. Viral growth kinetics were determined both with and without removing the residual inoculum after the adsorption period.

In the experiments wherein the inocula were not removed, virus growth was correlated with developing CPE. Those experiments were carried out in the following manner. A total of 6×10^6 log-phase cells of both TN-368-10 and TN-368-13 were each transferred to new 75-ml plastic cell culture flasks and allowed to attach to the plastic undisturbed for 2 h. The medium was then removed, and 2 ml of infectious cell culture medium at 6.0×10^7 PFU per ml was added to each flask (multiplicity of infection of 20). The flasks were rocked for 1 h on a Bellco rocker platform at a setting of 4 to enhance virus adsorption. After this period, 8 ml of fresh medium containing the above concentration of antibiotics and Fungizone was added to each flask. Beginning at the end of the adsorption period (zero time), 0.5-ml samples were removed at regular intervals. Before each sampling, the cells were made into a homogeneous suspension by gentle shaking and pipetting. The cells in the sample, then, were pelleted by centrifugation at $300 \times g$ for 5 min. The supernatant fluid was transferred to a new tube and was centrifuged at $2,100 \times g$ for 10 min before being carefully removed and frozen at −70°C for later titration. The cells in the $300 \times g$ pellets were resuspended, and 300 to 400 were inspected with a Zeiss phase microscope for signs of CPE. Those cells showing signs of CPE were scored as being in the prepolyhedral or polyhedral stage (31). The experiments wherein the inocula were removed were done as follows. A total of 2×10^6 TN-368-10 log-phase cells were allowed to attach to a new 25-ml culture flask. After cell attachment, the medium was removed and 3.8×10^7 PFU in 0.5 ml of TNM-FH was added and allowed to adsorb for 1 h while being rocked. After the adsorption period, 4.5 ml of TNM-FH (with antibiotics and Fungizone) was added to the flask. The cells were suspended in 5 ml of medium and pelleted two more times at $300 \times g$ for 15 min before their final resuspension in 5 ml of medium. A sample was taken at this time (zero time) and periodically thereafter, in which the cells were removed by centrifugation and the supernatant fluid was stored at −70°C for later titration. A sample was removed also at zero time for determining the cell concentration.

Antisera. Antisera to gradient-purified Ro*M*NPV and Ac*M*NPV LOVAL and TN-368-13-derived PMB-NOV were generated in the following way. Rabbits (from which preimmune blood samples were taken) were injected intramuscularly in two sites as well as in each rear footpad with 100 μg of viral protein mixed with complete Freund adjuvant. Two weeks later they were injected intravenously with 50 μg of virus and were injected again in another 2 weeks. One week later they were test bled. Weekly after that they were both bled and injected intravenously with 50 μg of virus until termination. Antisera used in these experiments were from the second bleeding.

Virus neutralization. Dilutions of antisera were made in Grace medium, pH 7.5, with 0.5% bovine serum albumin and antibiotics. Virus samples were diluted in TNM-FH such that 0.1 ml of a 1:2 dilution would contain 100 to 200 PFU (8). Equal volumes (0.3 ml) of the prediluted antisera and the prediluted virus were mixed and allowed to incubate at either room temperature (28°C) or 37°C for 1 h. Samples (0.1 ml) were then plated to determine the remaining PFU by the plaque assay. For some experiments sera were heated to 56°C for 30 min to remove any heat-labile nonspecific inhibitors or enhancers of virus infection (8).

Adsorption of antisera. Fifty microliters of undiluted anti-Ac*M*NPV PMB-NOV was added to 1 ml of 240 μg of Ac*M*NPV LOVAL in 0.01 M Tris (pH 7.8)-0.01 M EDTA. This mixture was incubated at 37°C for 2 h with occasional vortexing and then incubated again at 4°C for 72 h. The suspension was then centrifuged at $100,000 \times g$ and 5°C for 1 h to remove the LOVAL. The supernatant fluid was carefully removed and used as a 1:20 dilution of adsorbed antiserum. When this serum was tested for residual LOVAL infectious activity, none was found.

RESULTS

Growth curves of infected and uninfected cells. Figure 1 shows growth curves of viable infected and uninfected TN-368-10 and TN-368-13 cells. It is evident that the cells ceased division very soon after infection and that very little, if any, cytolysis occurred before 48 h postinfection. The cell membranes before 48 h postinfection had not become permeable to molecules the size of trypan blue; thus the possibility of nonselective leakage of virus particles during that time is remote. A similar curve, obtained for infected *S. frugiperda* cells (not shown), revealed that limited cytolysis occurred between 24 and 48 h postinfection, making it necessary to collect PMB-NOV from these cultures at 24 h postinfection.

Virus growth curves. The cell lines used to

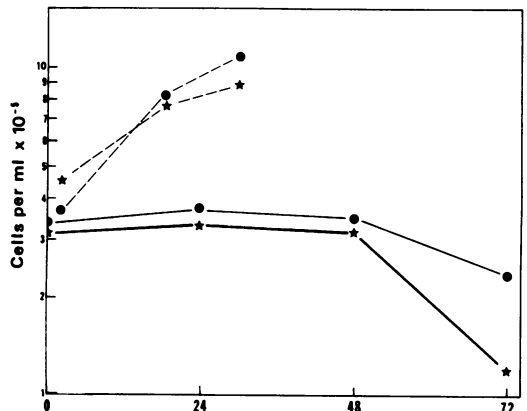

FIG. 1. *Growth curves of infected (——) and uninfected(- - -) TN-368-10 (★) and TN-368-13 (●) cells. Cell samples from infected and uninfected cultures were removed periodically, and viable cell counts were made as described in the text. For the infected cultures, the 0-h sample was taken at the end of the adsorption period.*

study extracellular Ac*M*NPV growth kinetics were TN-368, clones 10 and 13, because, as previously reported, clone 13 cells revealed only 25% of the PFU relative to clone 10 when used as indicator cells in the plaque assay (31). It was of interest to determine, therefore, whether the growth characteristics of Ac*M*NPV were similar in both cloned lines. In addition, we were curious to know whether a temporal relationship between PMB-NOV and CPE could be established. The results of viral growth studies (with the residual inocula not removed) plotted with the corresponding changes in CPE are shown in Fig. 2. With regard to TN-368-10 and TN-368-13, the results obtained were similar, with perhaps two exceptions. The rate of development of the polyhedral phase of CPE (and the rate of decline of the prepolyhedral phase) was slightly less in clone 13 cells than in clone 10 cells, and the concentration of extracellular PFU reached a slightly greater maximum with clone 13 cells than with clone 10 cells. Other aspects of the two curves were the same; in both cases extracellular virus began to increase at 8 h postinfection, coincident with the onset of the prepolyhedral stage of CPE in the cell population. Prepolyhedral CPE increased very sharply, progressing from 0 to approximately 80% of the cells in a 2-h period. The polyhedral stage of CPE began between 12 and 14 h postinfection and increased slightly less rapidly than the prepolyhedral stage. In both cultures the maximum PMB-NOV concentration was reached at 36 h postinfection, followed by a gradual decrease. It is interesting that a histogram representing the change in PMB-NOV concentration with time exactly coincided with the curve representing the percentage of cells with prepolyhedral CPE.

A second viral growth curve was done with the residual inoculum removed to reveal the exponential increase in PMB-NOV that was masked by the remaining inocula in the previous experiments (Fig. 3). During the centrifugation and rinsing procedure necessary to remove the residual inoculum, however, 25% of the cells were lost.

Physical-infectious particle ratio. The physical-infectious particle ratios for Ac*M*NPV LOVAL and PMB-NOV are given in Table 1. It was determined that Ac*M*NPV LOVAL had a physical-infectious particle ratio of about 2.2×10^5:1 for the bundles and 2.4×10^5:1 for the enveloped single nucleocapsids, as assayed by the plaque assay in vitro. In contrast to this, the Ac*M*NPV PMB-NOV had a physical-infec-

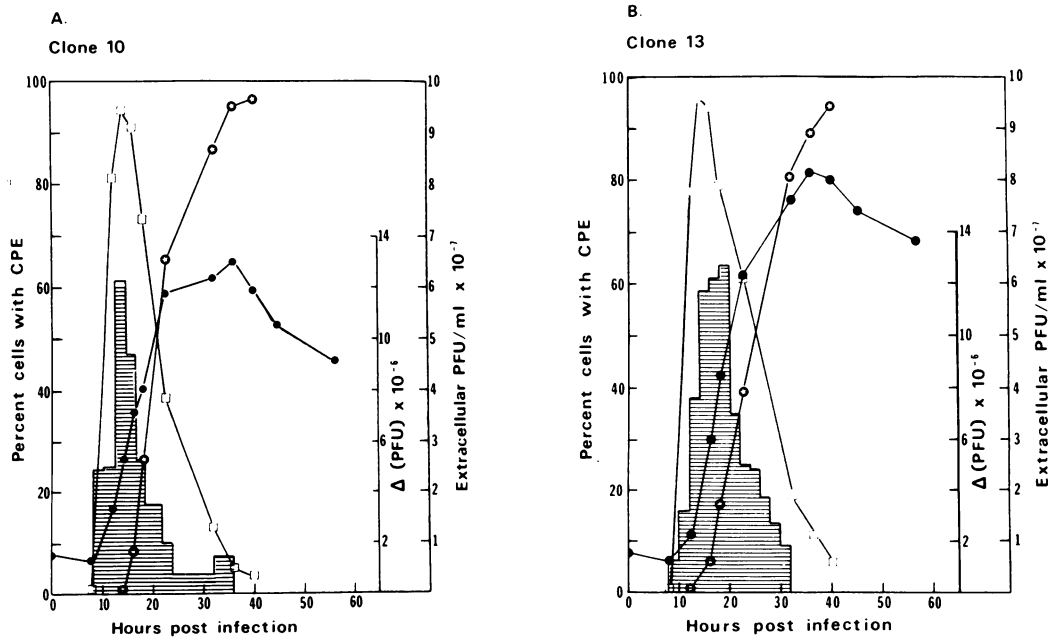

FIG. 2. *Growth curves of Ac*MNPV *PMB-NOV in TN-368-10 (A) and TN-368-13 (B) cells correlated with the progressive development of CPE. The appearance of infectious extracellular virus (●) was monitored by using a plaque assay, and the development of CPE, both the prepolyhedral stage (□) and the polyhedral stage (✪), was monitored visually by examining 300 to 400 cells at each sampling with a Zeiss phase microscope. The histogram (▤) represents the change in extracellular virus concentration with time. For details of the experiments, see text.*

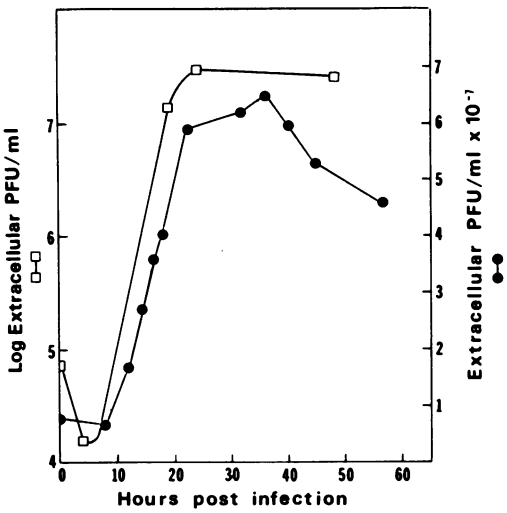

FIG. 3. *Comparison of growth curves of AcMNPV extracellular nonoccluded virus in TN-368-10 cells with (□) and without (●) the residual inoculum removed. For details, see text.*

tious particle ratio of about 128:1. If one can assume that the DNA-protein ratio is the same for the various forms of RoMNPV as it is for the analogous forms of AcMNPV, then the calculated physical-infectious particle ratios for these forms are very similar as well (1.6×10^5:1 for bundles; 2.9×10^5:1 for singles). To test whether the necessary exposure to alkaline conditions of the LOVAL preparations could account for their relatively greatly diminished infectivity, a sample of cell culture-derived extracellular nonoccluded AcMNPV was taken through the alkaline solubilization procedure and then tested for infectivity. The physical-infectious particle ratio was increased about twofold (to 256:1).

Virus neutralization. Antisera raised against AcMNPV and RoMNPV LOVAL were tested for neutralization activity against AcMNPV and RoMNPV from various sources. The results of these experiments were that the antisera neutralized the homologous and heterologous LOVAL (Fig. 4). It did not neutralize other forms of the virus, however, such as PMB-NOV, alkali-treated PMB-NOV, hemolymph-derived NOV, intracellular NOV from cultured cells, and intracellular NOV from the fat body (Table 2). It was evident by comparing the serum-free control values with the corresponding serum-containing control values that nonspecific factors present in both the immune and preimmune sera slightly inhibited the infectivity of LOVAL, but enhanced the infectivity of NOV. If the sera were heat inactivated at 56°C for 30 min, these effects were diminished. The nonspecific inhibition of LOVAL infectivity was eliminated altogether, whereas approximately a twofold enhancement in the infectivity of NOV was still observed at serum concentrations of 2.5×10^{-3} or greater.

Figures 5 through 11 show the neutralization activity of anti-AcMNPV PMB-NOV serum versus various different forms of AcMNPV and RoMNPV. Figure 5 shows the comparative curves of anti-AcMNPV LOVAL serum and anti-AcMNPV PMB-NOV serum (heat inactivated and untreated) versus AcMNPV LOVAL. The curves of the untreated sera were very nearly identical, indicating that the neutralization titer in the heterologous serum was equal to that of the homolgous serum, but when the sera were heat inactivated, the 50% neutralization titer of the homologous antisera (anti-AcMNPV LOVAL) was apparently increased (about fourfold), whereas the titer of the heterologous antisera appeared to be relatively unchanged.

Figure 6 depicts the neutralization capability of anti-AcMNPV PMB-NOV serum (heat inactivated and untreated) versus AcMNPV PMB-NOV, and the untreated serum versus both LOVAL and PMB-NOV of RoMNPV. Heat treatment of this serum again did not alter its neutralization properties, as shown here tested against the homologous form (PMB-NOV). It should be noted that the titer of this serum not only was identical with the comparable forms of both AcMNPV and RoMNPV, but was higher against the NOV than against the alkali-liberated viruses.

TABLE 1. *AcMNPV physical-infectious particle ratios as assayed in vitro*

Virus prepn	DNA		Particle/μg of protein (× 10^9)	PFU/μg of protein	Particles/PFU
	μg/μg of protein	g/μg of protein (× 10^{-7})			
PMB-NOV	0.19	1.9	1.1	8.9×10^6	1.28×10^2
LOVAL I[a]	0.20	2.0	1.2	5.0×10^3	2.4×10^5
LOVAL II-X[b]	0.30	3.0	1.8	8.4×10^3	2.2×10^5

[a] Single nucleocapsid per envelope.
[b] Many nucleocapsids per envelope.

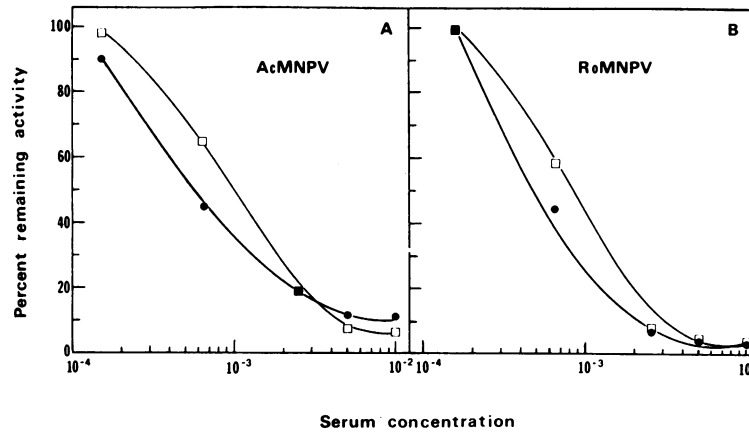

FIG. 4. *Neutralization of AcMNPV LOVAL (A) and RoMNPV LOVAL (B) with rabbit antisera generated against AcMNPV LOVAL (□) and RoMNPV LOVAL (●). Virus samples were incubated for 1 h at 37°C with the indicated concentration of antiserum, and then 0.1 ml was assayed for biological activity. (The concentration of undiluted antiserum is 1.) The 100% value of biological activity was the average of the control virus samples incubated with preimmune serum at the various concentrations. All assays were done in duplicate.*

Neutralization of intracellular NOV from both TN-368-13 cells and the fat body of *T. ni* larvae with heat-inactivated anti-Ac*M*NPV PMB-NOV (Fig. 7) indicated that the intracellular NOV shared a biologically important antigenic determinant(s) with PMB-NOV that was not present or accessible on LOVAL (Table 2).

Figure 8 shows the results of the neutralization of both Ac*M*NPV PMB-NOV and LOVAL with Ac*M*NPV LOVAL-adsorbed anti-Ac*M*NPV PMB-NOV. As can be seen, there was no remaining neutralization activity against LOVAL, whereas there was no change in the titer against the PMB-NOV. These results confirm the conclusion that the LOVAL and PMB-NOV forms are antigenically distinguishable.

Figures 9, 10, and 11 show the respective neutralization curves of the nonoccluded forms of Ac*M*NPV from infectious hemolymph, Ro*M*NPV from infectious hemolymph, and Ac*M*NPV PMB-NOV from *S. frugiperda* cells with anti-Ac*M*NPV PMB-NOV. Heat inactivation of the serum seemed to slightly increase the 50% neutralization titer against each of these forms (fourfold or less). Adsorption of this antiserum with Ac*M*NPV LOVAL likewise seemed to slightly increase the titer (Fig. 9; with others, data not shown), but adsorption of heat-inactivated serum with Ac*M*NPV LOVAL did not increase the titer further (Fig. 10 and 11).

DISCUSSION

Growth curves of infected and uninfected cells. The inhibition of cell division in Ac*M*NPV-infected TN-368-10 and TN-368-13 cultures was shown by comparing the cell concentration over a period of time in infected and uninfected cultures. Inhibition apparently occurred quite rapidly since there was no increase in cell concentration after the adsorption period. This inhibition of cell division upon infection with NPV has been noted before (2, 17, 21). That there was very little, if any, cytolysis before 48 h postinfection was indicated by the exclusion of trypan blue in cell samples counted in that period. Only after 48 h postinfection was there an increase in dye-stained TN-368 cells. Cells behaved similarly when infected with Ro*M*NPV.

Virus growth curves. In these studies, the increase of extracellular virus was monitored early in infection during a time of very little, if any, cytolysis. This fact assures us that we were measuring the increase of budded virus. We performed the growth studies in two ways, one in which the inoculum was removed after adsorption and one in which it was not removed. We did not remove the inocula in the latter studies because we wanted to correlate the sometimes subtle morphological changes in the infected cells with the increase in extracellular titer, and we were concerned that the repeated rinsings and centrifugations required to remove the inocula might alter the morphology, as well as injure and even lyse a significant percentage of the very fragile cells (at least 25% were lost when this type of experiment was done). But because of the residual inoculum, virus growth in these studies appeared to be only arithmetic. A study wherein

TABLE 2. *Neutralization activity of anti-LOVAL serum tested against various forms of RoMNPV and AcMNPV*

Antisera vs:	Virus type and source[a]	PFU at serum concn of:[b]						NS[c]
		10^{-2}		5×10^{-3}		2.5×10^{-3}		
		P	I	P	I	P	I	
AcMNPV LOVAL	AcMNPV LOVAL	96	0	114	16			125
AcMNPV LOVAL	AcMNPV Plasma membrane Budded Nonoccluded	60	80	77	90			35
AcMNPV LOVAL	AcMNPV Infectious Hemolymph	200	218	178	130			100
AcMNPV LOVAL	AcMNPV Homogenized Fat body	157	125	155	166			110
AcMNPV LOVAL	AcMNPV Culture derived Nonoccluded Intracellular	335	370	352	359			175
AcMNPV LOVAL	AcMNPV Alkali treated Plasma membrane Budded Nonoccluded	81	88			53	58	34
RoMNPV LOVAL	RoMNPV LOVAL	23	0	36	0			47
		121	9			113	50	130
RoMNPV LOVAL	RoMNPV Plasma membrane Budded Nonoccluded	44	46	34	33			16.5

[a] For explanations on the preparation of the various virus samples, see text.

[b] The concentration of undiluted serum is 1. The antisera for these experiments were not heat inactivated. Results indicate the mean number of PFU remaining in 0.1 ml of virus sample, as assayed in vitro, after having been incubated 1 h at 28°C with the indicated concentration of serum. P, Preimmune; I, immune.

[c] No serum present.

the inoculum was removed confirmed that growth of the virus was indeed exponential (Fig. 3).

It is interesting to compare these growth curves with those done in similar systems in other laboratories. Knudson and Tinsley (17) did a growth study of *S. frugiperda* MNPV in an *S. frugiperda* cell line and found a peak in extracellular virus at about 48 h postinfection and another at about 96 h postinfection. They stated that lateral transmission of infectivity occurred 1 to 2 days before any lysis was observed, but that cytolysis did occur 2 to 3 days postinfection. We suggest, then, that the early peak in their system was due to budded virus, as is ours, and that the latter peak was due to cytolysis and the consequent release of internal nonoccluded virions. Dougherty et al. (5) infected *S. frugiperda* IPRL-21 cells with AcMNPV and showed a maximum titer of extracellular NOV between 3 and 4 days postinfection. Knudson and Tinsley similarly reported that maximum titer was reached 4 days postinfection in their system. We submit that the extracellular virus population at this time is due to both budded virions and virus released as the consequence of cytolysis. Our maximum titer at 36 h postinfection, on the other hand, was a reflection of budded virus.

The most interesting aspect of the growth curves was that the prepolyhedral stage occurred simultaneously with the appearance of

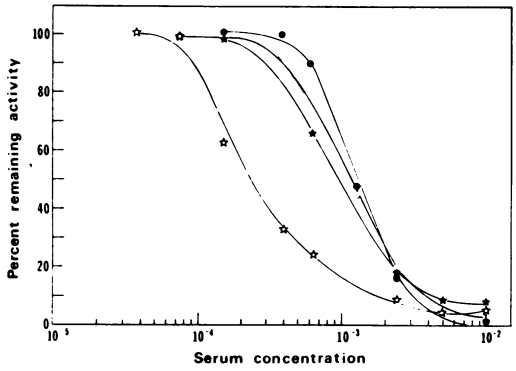

FIG. 5. *Neutralization of AcMNPV LOVAL with heat inactivated (☆) and untreated (★) anti-AcMNPV LOVAL serum, and with heat-inactivated (○) and untreated (●) anti-AcMNPV PMB-NOV serum. Neutralization was carried out as described in Fig. 4 and the text. Heat inactivation of antisera is described in the text.*

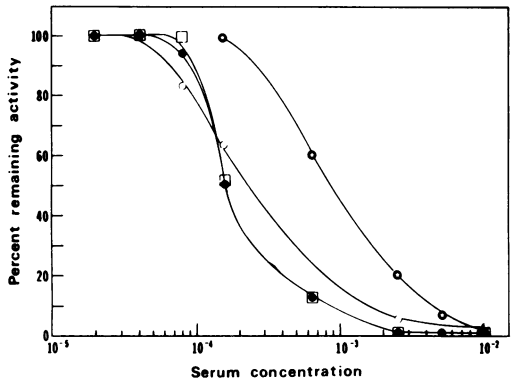

FIG. 6. *Virus neutralization with untreated anti-AcMNPV PMB-NOV serum of AcMNPV PMB-NOV (●), RoMNPV PMB-NOV (○), and RoMNPV LOVAL (✪). Heat inactivation of this antiserum did not alter the neutralization curve of AcMNPV PMB-NOV (□).*

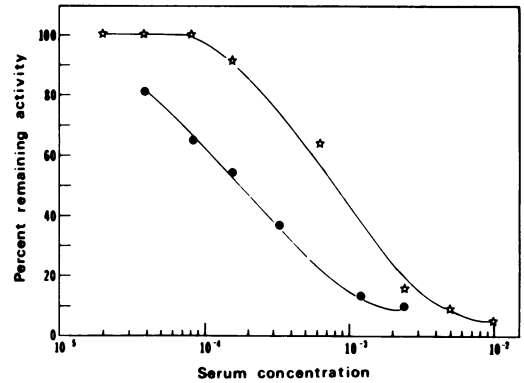

FIG. 7. *Neutralization of intracellular nonoccluded virus from both TN-368-13 cells (●) and the fat body of infected T. ni larvae (☆) with heat-inactivated anti-AcMNPV PMB-NOV serum. For experimental details, see Fig. 4 and text.*

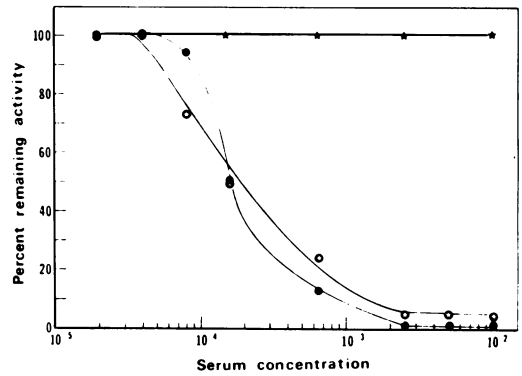

FIG. 8. *AcMNPV LOVAL-adsorbed anti-AcMNPV PMB-NOV serum versus AcMNPV PMB-NOV (✪) and LOVAL (★). The unadsorbed anti-AcMNPV PMB-NOV versus AcMNPV PMB-NOV curve is shown in comparison (●). The adsorption of antisera is described in the text. Neutralization was carried out as described in Fig. 4.*

extracellular virus, and that the increase in extracellular virus subsided with the progression of the prepolyhedral stage to the polyhedral stage. A possible interpretation of these data is that the formation of polyhedra, directly or indirectly, has an inhibitory effect on the budding of extracellular virus. Another possibility is that the two events are controlled by a common third event; i.e., whatever triggers the condensation of polyhedra also shuts down the budding of the virus through the plasma membrane. Whatever the cause, there definitely seems to be an inverse correlation between the budding of virus and the appearance of polyhedra. It is not surprising, then, that clone 13, which produced polyhedra slightly more slowly than clone 10, also produced slightly more extracellular virus. This difference in extracellular virus yield has been observed before (31).

Injac et al. (14) noted in their in vivo study of the release of NPV from *Hyphantria cunea* Drury larvae that the budding and release of NOV particles was an active process that occurred largely before intranuclear viral occlusion took place and was not the result of advanced infection and subsequent nuclear envelope breakdown, as has been suggested by some authors (19).

Raghow and Grace (21), in their study on the multiplication kinetics of NPV in *Bombyx mori*, showed that the onset of the exponential phase of virus production occurred simultaneously with the appearance of the precursor of

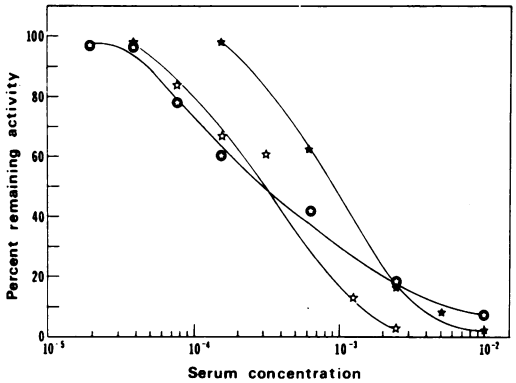

FIG. 9. *Neutralization of hemolymph-derived nonoccluded AcMNPV with anti-AcMNPV PMB-NOV serum: (★) untreated serum; (✪) serum adsorbed with AcMNPV LOVAL; (☆) heat-inactivated serum. For experimental details, see Fig. 4.*

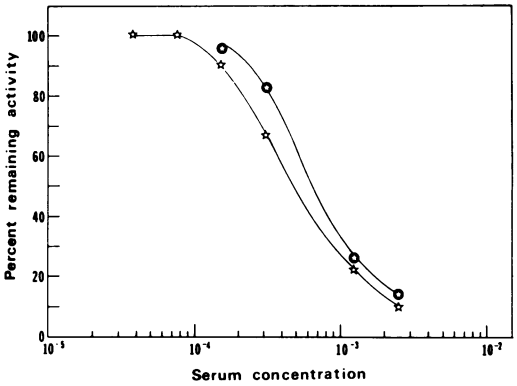

FIG. 10. *Neutralization of hemolymph-derived nonoccluded RoMNPV with anti-ACMNPV PMB-NOV serum: (☆) heat-inactivated serum; (✪) heat-inactivated, AcMNPV LOVAL-adsorbed serum. For experimental details, see Fig. 4.*

the virogenic stroma in the nucleus. Their method of viral assay, however, did not discriminate between occluded and nonoccluded budded virus, so there are no data comparable to ours concerning the shutdown of budding virus with the onset of polyhedron formation.

Physical-infectious particle ratio. The results indicated that for Ac*M*NPV LOVAL only 1 in 2.2 × 10^5 particles (bundles) or 1 in 2.4 × 10^5 particles (singles) is infectious in cell culture, whereas 1 in 1.28 × 10^2 particles of PMB-NOV is infectious. Knudson and Tinsley (17) estimated the physical particle-infectious unit ratio of *S. frugiperda* extracellular nonoccluded NPV (by the latex sphere-electron microscope method) to be 62 to 310 particles per mean tissue culture infectious dose ($TCID_{50}$). They also showed that their system did not

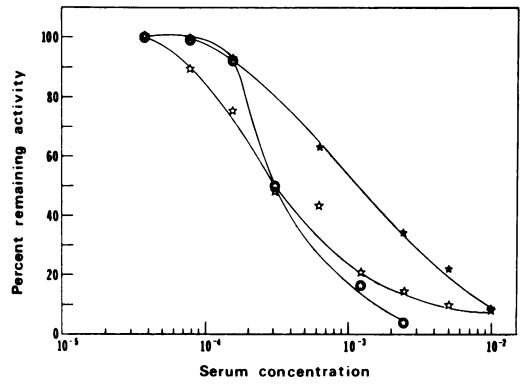

FIG. 11. *Neutralization of AcMNPV PMB-NOV grown in Sodoptera frugiperda cells with anti-AcMNPV PMB-NOV serum: (★) untreated serum; (☆) heat-inactivated serum; (✪) heat-inactivated, AcMNPV LOVAL-adsorbed serum. For experimental details, see Fig. 4 and text.*

deviate from the expected Poisson distribution when assaying infectivity, and thus 0.7 PFU was equivalent to 1 $TCID_{50}$. Using this value and converting $TCID_{50}$ to PFU, their estimate was that 1 in 99 to 443 particles was infectious in cell culture. This value is in close agreement with ours for PMB-NOV.

It was of interest to determine whether the 1,700- to 1,900-fold difference in infectivity between the LOVAL and PMB-NOV forms of the virus could be attributed to LOVAL's necessary exposure to alkaline conditions. The results clearly show that exposure to alkali is not solely responsible for the apparent dearth in infectivity of the LOVAL preparations, since the same exposure to PMB-NOV only reduced the infectivity twofold, such that 1 in 256 was infectious. In a similar experiment, Vaughn and Faulkner (30) exposed infectious hemolymph to alkali and found that the treatment did not appreciably affect the biological activity. Further, Dougherty et al. (5) reported that nonoccluded Ac*M*NPV derived from cell culture and infectious hemolymph were noninfectious when injected per os into *S. frugiperda* larvae, but were infectious when injected into the hemocoel. Kawarabata (15), on the other hand, reported that hemolymph-derived NOV from *B. mori* is 100-fold more infectious by hemocoelic inoculation than the corresponding LOVAL, but that by per os injection the LOVAL was more infectious than the NOV. All these data indicate that the difference in infectivity of the LOVAL and the NOV in tissue culture, as well as by the different routes of injection in vivo, is due not to experimental manipulation, but to actual differences in the viral forms, which

most likely reflects their respective varied specificities.

It has been suggested that, in nature, the nonoccluded forms of the virus are responsible for the infection of secondary tissues and organs whereas the occluded forms initiate the infection (9, 19, 24, 25). This idea agrees with the observations that NOV are more infectious by the hemocoelic route of infection and that LOVAL is more infectious by the oral route. It is consistent, too, with the observation that the NOV are more infectious in cultures of ovarian cells, such as the TN-368 and *S. frugiperda* lines, than is LOVAL.

Virus neutralization. The results of the neutralization tests showed than anti-Ro*M*NPV and anti-Ac*M*NPV LOVAL sera neutralize both the homologous and heterologous LOVAL forms of the virus, but neither will neutralize even the homologous nonoccluded forms of the virus. These results suggest that antigens important in biological activity are shared in the in vitro infectious LOVAL of two different viruses, but either are not present or are not exposed in the infectious forms of the NOV, or are not important in the expression of their biological activity. In any case, the two forms of in vitro infectious virus appear to be antigenically if not biologically different, whereas the same forms of two different viruses, Ac*M*NPV and Ro*M*NPV, share biologically important antigenic determinants.

The reciprocal tests, i.e., neutralization of the various forms with anti-Ac*M*NPV PMB-NOV, showed that all forms tested of both Ac*M*NPV and Ro*M*NPV could be neutralized, but that the titer was generally higher against the nonoccluded forms than the alkaline-liberated forms, especially if the serum was heat inactivated. Adsorption of anti-Ac*M*NPV PMB-NOV with Ac*M*NPV LOVAL completely removed all neutralization activity against Ac*M*NPV LOVAL, whereas the titer against Ac*M*NPV PMB-NOV and other nonoccluded forms was undiminished. These results confirm the initial findings that those antigens important in the neutralization of LOVAL and PMB-NOV are different. They also indicate that whereas LOVAL does not possess an accessible antigen similar to the ones important for the neutralization of PMB-NOV, PMB-NOV does share the antigen that is active in the neutralization of LOVAL. This lack of reciprocity can be accounted for in several ways. First, if neutralization involves the alteration or masking of receptor sites, then even though PMB-NOV shares a receptor site with LOVAL, it apparently has an alternate site that is used preferably. This explanation would be consistent with the observation that PMB-NOV is 2,000-fold more infectious than LOVAL in vitro, and this difference in infectivity could be due to an additional, more efficient specific receptor site on PMB-NOV.

An alternative hypothesis is that the envelope present on LOVAL is inhibitory to infection in vitro and that only the few unenveloped nucleocapsids in a mostly enveloped preparation are infectious, whereas in the more highly infectious PMB-NOV preparations the infectious agent is in fact the enveloped form. Assuming that the nucleocapsids of these two forms are identical and that only the envelopes are different, antisera made to PMB-NOV as well as LOVAL could contain anti-nucleocapsid activity that would neutralize the "infectious" nucleocapsids in the LOVAL preparations. This anti-nucleocapsid activity would not become evident in the neutralization of PMB-NOV, however, due to the greater infectivity of the enveloped forms as well as the greater concentration. If this hypothesis is true, anti-LOVAL activity should be removable from either antisera by adsorption with purified nucleocapsids. We have unsuccessfully attempted to do this with nucleocapsid preparations made from Nonidet P-40-exposed LOVAL. The experiments were not conclusive, however, in that no infectious activity was retained in these nucleocapsid preparations, and thus important antigens could have been lost or altered.

A third alternative in explanation of the anti-LOVAL activity in the PMB-NOV antisera is the possibility that the PMB-NOV used to immunize the rabbits were contaminated with LOVAL-like antigens from intranuclear occluded virus in the cultured cells in spite of our efforts to prevent it. Only further and more rigorous experimentation will reveal which, if any, of these hypotheses is correct.

The results also indicated that antigens important in the neutralization of NOV other than PMB-NOV are present and antigenic on PMB-NOV. That the same antigen is active in the neutralization of all forms of NOV, however, has not been determined. The enhancement of the neutralization titer of untreated Ac*M*NPV PMB-NOV antisera against alternate nonoccluded forms by its adsorption with Ac*M*NPV LOVAL indicates that the LOVAL is removing some component(s) of the serum that interferes with the neutralization of the alternate nonoccluded forms (Fig. 9). This same component(s) does not interfere with the neutralization of PMB-NOV, however (Fig. 8). That this enhancement is also achieved by heat inactivation of the serum (Fig. 9–11), and that heat inactivation and adsorption both does not

have an additive effect, indicates that the component(s) is a nonspecific heat-labile factor. Even though in some instances the titers of heat-inactivated sera were slightly higher than those of the untreated sera (Fig. 5, 9–11), the overall results of the neutralization experiments, i.e., the presence or absence of neutralization activity, were largely independent of heat treatment. It was curious, however, that nonspecific heat-labile factors present in the antisera and preimmune sera had the opposite effects on the infectivity of NOV and LOVAL. The infectivity of NOV was enhanced in the presence of these factors, whereas the infectivity of LOVAL was diminished (Table 2). That the effects were opposite only indicates another way in which LOVAL and NOV are different.

Another curious observation is that heat inactivation of anti-Ac*M*NPV LOVAL serum seemed to increase the titer of the serum against Ac*M*NPV LOVAL (Fig. 5), yet it abolished the heat-labile inhibitory activity seen in the untreated serum. We can offer no good explanation for this observation but suggest that it is possible that the untreated serum could contain heat-labile factors that sterically interfere with the immunological neutralization of LOVAL, and that these factors, along with the nonspecific inhibitory factors, are destroyed by heat treatment.

No experiments were included in this report involving culture-derived occluded virus because we have encountered some difficulty in obtaining highly purified virus from this source. We are continuing efforts to obtain these preparations, however.

The neutralization results presented in this report show rather significant antigenic differences between LOVAL, PMB-NOV, and other NOV, and subtle differences between PMB-NOV and other NOV. These antigenic differences could very possibly be attributable to envelope differences. Viral antigenic differences due to envelope sources is not unique to the NPVs; this situation exists also for the poxviruses. Although at least 90% of the progeny in poxvirus infections are presumed to gain their membranes by de novo synthesis in the host cell cytoplasm and remain intracellular (4), 10% or less are released into the medium by budding through the plasma membrane (6, 7, 20, 22). Not only are these budded virions less dense than the intracellular virions (1), as are the PMB-NOV of Ac*M*NPV and Ro*M*NPV (29), but they are antigenically different (1). Antisera prepared to, and which will neutralize, the intracellular form of the virion will not neutralize the extracellular form.

The results presented in this paper show that there are significant differences between the alkali-liberated and nonoccluded forms of the NPVs of *R. ou* and *A. californica*, not only with regard to infectivity and antigenicity, but also in the timing of the virus maturation. The budding of PMB-NOV stops as the occlusion of intranuclear virus begins. These results, along with the mounting data indicating significant biochemical, biophysical, and morphological differences between the alkali-liberated and nonoccluded virions (29; Summers et al., manuscript in preparation), suggest structural differences that are indicative of differences in specificity, function, and role in the continuance of the infection cycle.

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